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Biomarkers of effect in endocrine disruption: how to link a functional assay to an adverse outcome pathway

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Abstract

The development of *in vitro* testing strategies may achieve a cost-effective generation of comprehensive datasets on a large number of chemicals, according to the requirements of the European Regulation REACH. Much emphasis is placed on *in vitro* methods based on subcellular mechanisms (*e.g.,* nuclear receptor interaction), but it is necessary to define the predictive value of molecular or biochemical changes within an adverse outcome pathway (AOP). AOP pivots on the description of the flow from a molecular initiating event through a cascade of intermediate events needed to produce a specific adverse effect at organism level: downstream responses at cell level are, therefore, essential to define an AOP. Several *in vitro* assays are based on human cell lines representative of endocrine-targeted tissues (*e.g.*, prostate) and on functional biomarkers of clinical relevance (*e.g.*, PSA secretion in human prostate epithelial cells). We discuss the implementation of such functional biomarkers in the AOP context.

Key words

- endocrine disruptor
- *in vitro* assay
- risk assessment
- toxicology
- testing strategy

Chemical risk assessment addresses the probability that a certain exposure level to a chemical can cause an adverse effect, whose nature and degree of severity, including possible reversibility, should be evaluated. The general, internationally accepted WHO/IPCS (World Health Organization/International Programme on Chemical Safety) definition of adverse effect is *"a change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub) population that results in an impairment of functional capacity or of the capacity to compensate for additional stress, or an increase in susceptibility to other influences"* [1 and refs therein]. This definition appears straightforward; indeed, it can still hold true in the changing framework of toxicology *(Figure 1A)*, where increasing attention is paid to the effects of molecular/biochemical mechanisms impinging on adverse outcome pathways (AOPs) [2, 3]. Many substances do have mechanisms that are readily connected with adverse effects in tissues and organisms, such as the formation of covalent adducts with DNA (*e.g.*, acrylamide) or the inhibition of key enzymes of the nervous system (*e.g.*, cholinesterase-inhibiting pesticides). The nature and dose-response of effects related to such mechanisms can be identified by the regulatory toxicity studies used to derive the values (*e.g.*, NOAEL, Benchmark Dose) which form the current basis of risk assessment. With the exception of genotoxicity, the current toxicological risk assessment is conceptually based on the existence of a threshold level for a given toxicological effect: an appreciable adverse effect will not occur below such threshold, even in the most susceptible population groups. To date the definition of a threshold mainly relies on *in vivo* tests performed according to international standardized test guidelines (TG), whose outcomes are based on changes of apical endpoints (*i.e.,* weight gain, reproductive performance, organ pathology, etc.). In most cases, testing and evaluation of a chemical are performed independently of any knowledge on its mechanism or mode of action (MoA) *(Figure 1A)* [5]. Within such scheme, mechanistic data may fulfill important specific purposes, from screening among chemical analogues through to biomarker identification assessing the human relevance of observed effects; however, knowledge of mechanism still is more often a supportive, rather than a fundamental, component of the toxicological assessment scheme.

In other instances, the matter may be more tricky, like in the intensively debated issue of endocrine disruptors (EDs) [4-6]. Let's consider tests that detect an "estrogenic/antiestrogenic" action, like the *in vitro* estrogen receptor (ER) transactivation assays (OECD TG-455 and -457) (OECD: Organization for Economic Cooperation and Development) or the *in vivo* uterotrophic assay (OECD TG-440). A viewpoint hold that a positive response to these tests merely poins out an endocrine-like MoA, whereas the adversity needs to be addressed

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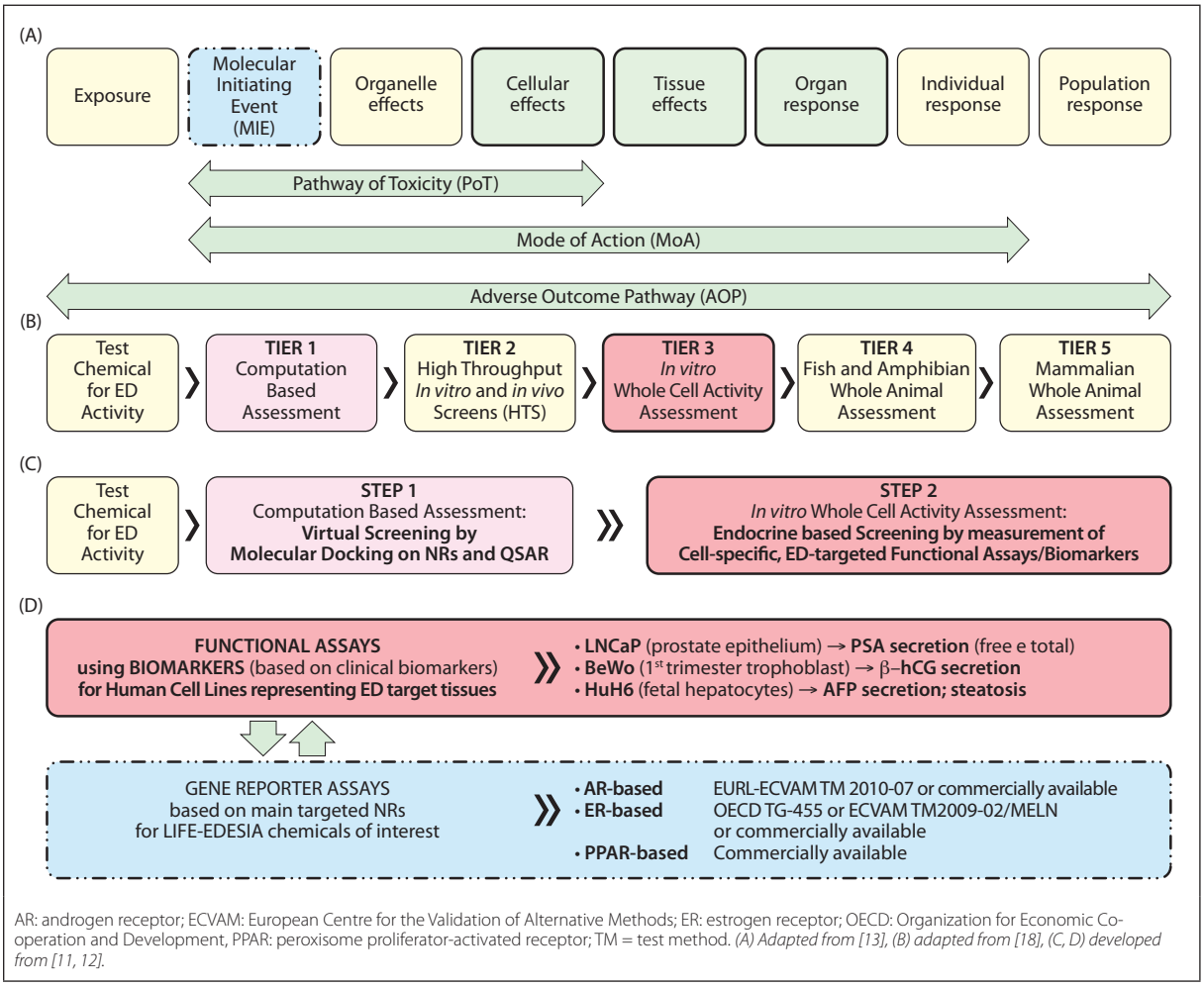


AR: androgen receptor; ECVAM: European Centre for the Validation of Alternative Methods; ER: estrogen receptor; OECD: Organization for Economic Co-operation and Development, PPAR: peroxisome proliferator-activated receptor; TM = test method. *(A) Adapted from [13], (B) adapted from [18], (C, D) developed from [11, 12].*

Figure 1
A comparison among the current views of the adverse outcome pathway (AOP), including mode of action and pathway of toxicity (A), the TiPED Tiered Protocol for Endocrine Disruption (B), and the LIFE-EDESIA animal-free Endocrine Disruptor (ED) screening strategy (C, D). LIFE-EDESIA *in silico-in vitro* ED screening strategy starts from a virtual screening (C) to proceed through the use of multiple cell-specific, ED-targeted, functional assays making use of biomarkers of effect derived from clinical biomarkers (D).

only by assessing apical effects. Some authors insist that "*endocrine disruption is just a mode-of-action that may or may not result in adverse effects*" and that it has to be dealt with like other non-genotoxic agents [4]. According to this viewpoint, endocrine disruption is somewhat like "*much ado for nothing*" because of endocrine activities falling mainly within the maintainance of the physiological homeostasis; in most cases, effects that matter are those identified by the conventional apical endpoints of *in vivo* assays. The straight application of this viewpoint might be pushed quite far away: a reduction of spermatogenesis without a demonstrated reduced fertility in laboratory animals or an altered brain biochemistry without proven neurobehavioural disturbances could be questioned with regards of their actual adversity.

An opposite position retains that pointing out an endocrine-like MoA indicates *per se* a potential hazard because of the critical importance of altered endocrine homeostasis during vulnerable life stages (*i.e.*, pregnancy, foetal development, puberty). Hence, small changes in hormone signalling can be compensated in the adult organism; changes of the same magnitude may however lead to adverse consequences when they occur in susceptible developmental windows [6, 7]. This second viewpoint questions the practical definition of a threshold, especially for chemicals interacting with nuclear receptors (NRs), thus acting through hormone mimicry or antagonism. It assumes that the NR interaction is maybe a toxicologically relevant initiating event; in such a case, a threshold likely exists but it is very low and difficult to determine. In addition, the threshold can be greatly different according to the diverse susceptibility of the organism life stages. Moreover, different exposure levels may have qualitatively different MoA and effects: for instance, low exposures might elicit endocrine (hormone-like or hormone-antagonist) effects, while more conventional toxic effects (*e.g.*, enzyme inhibition) may appear at higher dose levels. The dose-response curves for qualitatively different effects may overlap along the range of different exposure levels; this may explain the non-monotonic dose-response (NMDR) relationships that have been consistently reported in several *in vitro*

and *in vivo* studies on EDs [7]. The position emphasizing the toxicological novelty and significance of endocrine-like mechanisms appears more consistent with the advance in knowledge of molecular and cellular endocrinologyas well as with the majority of findings in toxicology. However, an exclusive focus on molecular mechanisms of ED may bring the risk of "*drowning into complexity*", as it points out a relevant issue without a prior identification of the proper tools to cope with it in risk assessment.

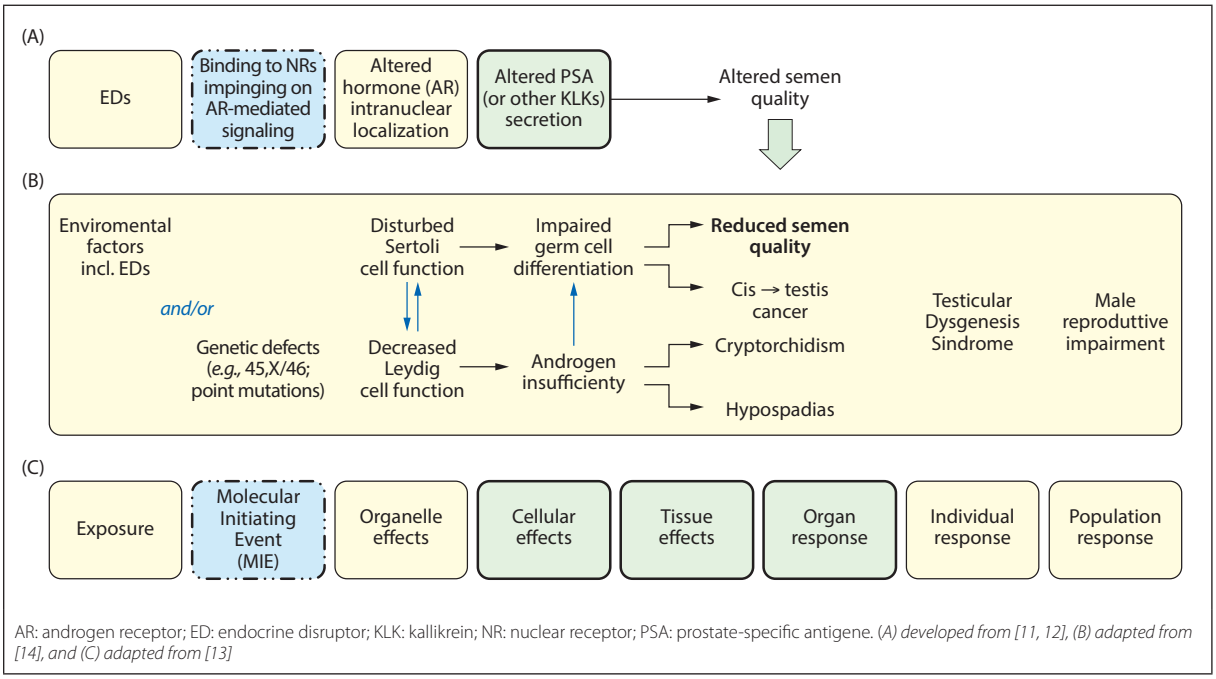
The development and application of hazard identification strategies exploiting new *in vitro* assays may achieve a cost-effective generation of comprehensive hazard data sets on a large number of chemicals: this will meet the general aim of the European Regulation REACH (2006/1907/EC) as well as major issues in the field of food safety (emerging contaminants, residues and environmental by-products of pesticides, etc.). However, such development has to face the critical challenge posed by the current *in vivo* testing framework based on toxic (biochemical/functional/morphological) effects in whole organisms. The challenge requires to build integrated testing strategies (ITSs) able to establish a robust link between a molecular or biochemical change and an adverse effect as defined according to [1]. In more detail, such link needs being based on the assessment of the predictive potential and the application domain of early changes at subcellular level, observed *in vitro*, toward an health-relevant outcome in the whole organism. Indeed, the same problem has been faced since decades by human medicine, where gene/protein expression or enzyme activities have been investigated as early diagnostic/prognostic predictors of chronic diseases, such as cancers; coping with this issues has delivered a number of biomarkers used in clinical practice.

The earlier proposal of a MoA framework *(Figure 1A)* started from the statement that "*an adverse ...effect can be described by a series of causally linked biochemical or biological key events that result in a pathological endpoint or disease outcome"* [8]. Hence, the AOP framework *(Figure 1A)*, initially proposed by the computational toxicology community, took place [2, 3]. The AOP concept pivots on the link of a chemical with a described pathway that leads to an adverse human health or ecological outcome, which is determined by the chemical's ability to trigger a molecular initiating event (MIE) *(Figure 1A)*. The MIE is just one, albeit necessary, component of the AOP, which builds up through a cascade of intermediate or key events at the subcellular, cellular, tissue and/or organ level leading to a specific adverse outcome (AO) at the individual or population level [3]. Another related framework is that of pathways of toxicity (PoT) *(Figure 1A)*, where the description of toxicological processes tends to focus on early events at the molecular and cellular level [9]. Overall, the AOP framework seems the most comprehensive, making up a flow of information from molecular through to *in vivo* and human data [10 and its Figure 3]. Indeed, AOP can be approached bottom-up – from the MIE to an AO – or top-down – from the pathogenesis of a human disease as an AO to the search for chemicals eliciting the relevant MIE.

So far, OECD TGs to screen ED-like activities are concentrated on MIEs overlooking the bottom-up responses (organelle and/or cellular ones) that represent the initial steps of both a MoA and AOP *(Figure 1A)*. To fill this gap, functional assays such as those developed and/or under development within different EU-granted projects (*e.g.,* ReProTect, SEURAT-1, LIFE-EDESIA) [11-13 and refs therein] might be the missing link to connect mechanistic endpoints to well-defined and measurable *in vitro* endpoints that are directly relevant to *in vivo* adverse effects. In addition, functional assays exploiting clinically-relevant biomarkers of effect, would greatly help the (practically difficult, albeit much voiced) design of animal-free ITSs to screen and characterize ED effects *(Figures 1C-D and 2A)*. An example of the potential contribution of cell-based functional assays measuring clinically-relavant biomarkers in human *in vitro* cell lines is here summarized and compared to an androgen-related AOP, namely the Testicular Dysgenesis Syndrome (TDS) [14]. As shown in *Figure 2*, since PSA secretion is androgen (DHT)-regulated, an androgen-like chemical affecting the secretion of PSA (or other androgen-regulated kallikreins/KLKs in prostatic fluid) have to trigger a MIE interacting with the AR-mediated signalling pathway either directly by AR binding or indirectly. An indirect MIE may occur via interactions with other NRs cross-talking with AR (*e.g.,* ERs, the aryl hydrocarbon receptor AhR) or with AR-cofactors [11 and refs therein]. With both a direct or an indirect MIE, the cellular effect (*i.e.* the change in PSA secretion) depends on the intermediate events occurring at the organelle level: for instance, in LNCaP cell line the intracellular localization of the ED-activated AR is different when different anti-androgens are used. In comparison to DHT, indeed, man-made chemicals (*e.g.,* pesticides) showed an increased AR nuclear localization whereas plant bioactives (*e.g.,* flavonoids) an increased AR microsomal localization [15, 16]. Overall, the ED-activated AR leading to decreased PSA secretion in LNCaP cells constitutes an alternative trigger of a MoA or AOP resulting, as tissue effect and organ response, in a reduced semen quality, that is one TDS component. The proposed functional assay brings in the contribution of accessory glands in the ED role on male infertility, thus integrating the TDS concept, that pivots on the effects on spermatogenesis [12].

LIFE-EDESIA (LIFE12 ENV/IT/000633; www.iss.it/life), a project granted within the frame of the EU LIFE Environment program, aims to demonstrate the feasibility of a animal-free, *in silico-in vitro* testing approach *(Figures 1C, 2A)* to search for alternative compounds to EDs classified (or suspected to be) as Substances of Very High Concern (SVHC; art. 57 of the REACH Regulation), but still widely used, the plasticizers phthalates and bisphenols. In addition LIFE-EDESIA considers also parabens, antimicrobial preservatives widespread used in cosmetics, pharmaceuticals and food, and suspected to have an ER-modulating relevance [17].

The research strategy is based on a tiered approach: i) screening a subset of already existing or *de-novo* designed alternatives by different *in silico* methodologies *(Figure 1C)*;



AR: androgen receptor; ED: endocrine disruptor; KLK: kallikrein; NR: nuclear receptor; PSA: prostate-specific antigene. *(A) developed from [11, 12], (B) adapted from [14], and (C) adapted from [13]*

Figure 2
Integrating the LIFE-EDESIA endocrine-based screening (A) using cell-specific, ED-targeted functional assays and biomarkers within the frame of the testicular dysgenesis syndrome (B) as an adverse outcome pathway (A).

ii) testing the *in silico* selected alternatives for their endocrine disrupting effects in three different human cell lines representative of endocrine-targeted tissues *(Figure 1D)*;
iii) assessing the applicability of the *in silico-in vitro* selected alternatives in prototypes of widely used consumers' products.

Namely, the potential alternative chemicals are first screened by a set of *in silico* methodologies, from chemico-physical to NR binding properties, from molecular docking to quantitative-structure activity relationship (QSAR) *(Figure 1C)*. In the ensuing step, chemicals passing the *in silico* screens are evaluated *in vitro* for their endocrine disrupting effects in three different human cell lines (trophoblast-like BeWo cells, fetal HuH6 hepatocytes and LNCaP prostate epithelial cells) representative of endocrine-targeted human tissues. ED-relevant effects are assessed by measuring cell-specific, biomarkers of effect *(Figures 1C-D)* that show the cellular response upon exposure to EDs in terms of cell function [11, 12 and refs therein].

The cell-specific, functional endpoints are toxicologically adapted clinical biomarkers, namely the secretion of proteins directly associated to the proper functioning of the secreting cells: in the case of trophoblasts, fetal hepatocytes and prostate epithelial cells, they are β-hCG (β subunit of the human chorionic gonadothropin), AFP (α-fetoprotein) and PSA (prostate-specific antigen), respectively (www.iss.it/life) (*Figure 1D*). Noticeably, this experimental approach largely overlaps with a recently proposed Tiered Protocol for Endocrine Disruption (TiPED) (*Figure 1B*) [18] aimed to support green chemistry in the design of new and less hazardous chemicals.

The LIFE-EDESIA strategy ultimately implements an AOP-derived approach from *in silico* NR binding through to clinically relevant functional biomarkers of endocrine disruption.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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